Cytokine production and measurement biology essay

Science, Biology

http://cheriburns1. files. wordpress. com/2011/11/uwslogo. jpgLINUS ORIE AWAHB00199899GCUREGULATION OF PAR2 AND AHR EXPRESSION IN HUMAN INNATE EFFECTOR CELLS. SUPERVISOR:- PROF. JOHN LOCKHARTABSTRACTBoth PAR-2 and AHR has been the subject of intensive research over the past decades. Both factors have been shown to be involved in the inflammatory response in both the innate and adaptive immune system. This study was undertaken to examine PAR2 expression and to determine whether AHR agonist TCDD has an effect on PAR-2 regulation and cytokine production to investigate the hypothesis that there is a link between PAR2 and AHR in innate macrophage effector functions. According to the results collected, monocytes were shown to express PAR-2 but no AHR detection due to the fact that antibody used does not recognise human AHR. And the cytokine production of IL-6 and TNFα of the human cell lines THP1 and U937 was inhibited by AHR agonist TCDD in a dose dependent manner. In conclusion, these findings identify that there is no link between PAR2 expression and AHR in both differentiated and non-differentiated human innate cells.

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1. INTRODUCTION

PAR2 and AHR has been the subject of intensive research over the past few decades. Research findings have been discovered which show the roles that each play in the innate immune system and adaptive immune system. They

also contribute to inflammatory response of major autoimmune disease such as rheumatoid arthritis.

1. 1Protease Activated Receptors (PAR2)

PAR is a membrane cell surface protein that belongs to the family of G-protein coupled receptor. They are highly expressed on the surface of cells and are activated by proteolysis of the N-terminus which reveals a tethered ligand sequence that interacts with the extracellular loop2 (Kelso et al, 2005: Macfarlane et al, 2001). There are four major types of PARs which are PAR1, PAR2, PAR3 and PAR4 but PAR2 has been shown to be implicated in inflammatory response of the immune system. During activation, PAR2 is resistant to thrombin but can be activated by trypsin, mast cells and tryptase. PAR2 activation has been shown to enhance the production and release of chemokine, cytokines and growth factors. This activated PAR signal different effector cells by different mechanism in the immune system (Shpacovitch et al, 2008: Soh et al, 2011). PAR2 is an up regulator of pro-inflammatory cytokines which support the evidence of the involvement of the receptor as a key regulator in innate immune system (Kelso et al, 2006).

1. 2Aryl Hydrocarbon Receptor

Aryl hydrocarbon receptor (AHR) is a ligand–dependent factor and member of the PAS superfamily of proteins. The immune system is a major target for AHR-mediated transcriptional regulation (Kerkvliet N. I, 2001: Stockingeret al, 2011)AHR is located on the cytoplasm. During the AHR activation in which the agonist (TCDD) binds to the cell. It translocate to the nucleus where it becomes active as a transcription factor. AHR activation is involved in

various physiological processes such as cell cycle regulation and differentiation (Stockinger et al, 2011). AHR mediates the toxicity of TCDD which is its most effective ligand. Previous studies have shown that macrophages are able to express AHR and also that exposure of innate immune cells such as monocytes and macrophages to dioxin (TCDD) displays an increase in cytokine production and AHR activation (Frericks et al, 2007: Cheon etal, 2007).

1. 3Innate immune cells (monocytes & macrophages)

Studies have shown that both the AHR and PAR2 play a major role in monocytes and macrophages. Monocytes act as the first line of defence in the innate immune system by attacking foreign antigens and also assist in the initiation of adaptive immune response (Martinez et al, 2012). Monocytes originate from myeloid cells. They circulate the blood stream for days and enter the tissue where it differentiates into macrophages. Monocytes derived macrophages have been shown to express PAR2 at both mRNA and protein levels and also AHR expression based on reaction of TCDD on cell numbers (Johansson et al, 2005: Kerkvliet, N. I 2009). Monocytes have been shown to secrete cytokines such as TNF-α and IL-6 (Martinez et al, 2012). TNF-α is recognized as mediating a wide variety of effector functions which includes cell activation and chemokine amplification. However, high levels of TNF-α production can lead to septic shock or organ damage. On the other hand, IL-6 is known as a B-cell regulator factor and mediates several functions on macrophages such as maturation and activation (Brennan et al, 2008). Monocytes are known to express toll-like receptors which are present on the

cell surface to recognize product of extracellular microbes. Increase in activation of TLR initiates various signalling pathways leading to the production of pro-inflammatory cytokines (Kerkvliet, N. I 2009: Huang et al, 2007).

1. 4Hypothesis

The hypothesis which is to be addressed in these study is to demonstrate whether a link between PAR2 regulation and AHR expressionexist by looking at these factors on the expression of human monocytes. The reason to investigate is to find out whether the innate protease sensor expressed by these cells and immune response such as cytokine production is regulated by an environmental influence.

2. EXPERIMENTAL MATERIALS AND METHODS

2. 1 Antibodies, reagents and equipment

Raisin Pipet-Lite multichannel pipette, Cornig Costar 96 well plates, Raisin Pipet-Lite pipettes, Raisin pipette tips, Falcon express (automatic pipette), Costar pipettes (10ml), Greneir blue capped tubes (50ml), Cornig centrifuge tubes (15ml), Grant Bio PMS-1000 shaker, Dispo-Safe P. E. T jars, Faster ultra safe cabinet, New Brunswick Galaxy 170R incubator (5. 0% CO2, 37oc), Neubauer improved haemocytometer, ENM handheld cell counter, IKA lab dancer, Jencons micro-centrifuge, Nikon Eclipse TS100 compound microscope, Hettich Zentrifugen Universal 320R centrifuge, Heraeus giofuge Primo-R centrifuge, Hettich Zentrifugen micro 200R centrifuge, CM scientific -80oc freezer, Greiner Bio-one cellstar. 12 well plate, Costar 24 well cell culture cluster plate, Fume hood, Nikon ELWD Microscope (magnification

x100), Dynex Magellan Biosciences (MRX ELISA reader). Anti-Aryl hydrocarbon Receptor antibody RPT1 (Abcam, UK), Phosphate buffered saline (Pro-Lab), Tween 20 (Sigma), Temed (Sigma), Virkon (10%), Ethanol (70%), Tryphan Blue, Heparin sodium (Leo Laboratories ltd), Histopaque-1077 (Sigma), RPMI Medium 1640 (Gibco), Glutamine, Penicillin, BD perm/wash buffer (BD biosciences), BD cytofix/cytoperm (BD biosciences), Albumin BSA (Sigma), Stabilized chromogen TMB (Invitrogen), LPS, 2, 3, 7, 8 Tetrachlorodibenzo-P-dioxin (TCDD, SuperCo), PMA/Ionomycin, IgG isotype antibody (Alexafloura Santa Cruz), Anti-mouse AHR PE FACS, AHR mouse monoclonal antibody

Preparation & isolation of peripheral blood mononuclear cell (PBMC).

Blood samples were taken from healthy volunteers through venepuncture and collected in heparin containing tubes (200ul/25ml of blood). The blood samples were diluted with PBS and blood cells were by Density Gradient Centrifugation (12000rpm, 20mins, 4oc) using 4ml of Histopaque (Sigma Laboratories). After Centrifugation, the plasma at the top layer was collected and used as a block for the FACS analysis. The white blood cells was taken out and washed with PBS in a fresh tube (12000rpm, 10mins, 4oc). Cells were counted with haemocytometer (Neubauer, Germany) and adjusted to 7. 5 x 106 ml that was later on used for both FACS analysis and Cell Culturing.

CD14 monocytes purification

From a blood sample, CD14+ cells were purified from the PBMCs and isolated using the Human CD14+ selection kit from Stemcell Technologies

(UK) according to the manufacturer’s instructions. The cell concentration was cultured at 2. 5 x 105ml and incubated (5oc, Co2, 37oc) for further experiments such as cell differentiation and ELISA procedure (Invitrogen, UK).

Cell lines culture

The human cell line U-937 and Monocytic leukaemia cell line THP-1 were maintained and cultured in RPMI 1640 containing L-glutamine, penicillin and 10% FCS (Foetal Calf serum) which was supplied by Invitrogen (UK).

Cell Differentiation and Stimulation

Both U937 and THP1 human macrophage were cultured in 96 well plates and 24 well plates in RPMI 1640 containing L-glutamine, penicillin and 10% FCS (Foetal Calf serum) with 150nm PMA for 3 days to enable differentiation of cells and cells were stimulated by the addition of LPS (100ng/ml) and TCDD (100nm) for cytokine production. Purified CD14+ cells (2. 5 x 106 ml) were cultured in MCSF (10ug/ml) and GMCSF (20ug/ml) for 4 days (37oc, 5% CO2) in RPMI 1640 media and differentiated with 150nm PMA/ionomycin. They were stimulated further for cytokine production by LPS (100ng/ml) and TCDD(100nm)

Flow Cytometry

Both U937 and THP1 were treated with medium and washed with PBS four times. Afterwards, the cells were washed by centrifugation (12000rpm, 5mins, 4oc) and blocked in FACS buffer (PBS/1% FCS/ 8ul/ml of 1% human plasma). The cells was washed again in Fix/Perm buffer to open cell for

staining through centrifugation and incubated with FITC conjugated anti-PAR2 (SAM 11) and Aryl Hydrocarbon receptor RPT1 (Abcam, UK) for 30 minutes at 4oc. The cells were resuspended in FACS buffer to be analysed in FACS Calibur (BD) and FLowJO software.

Cytokine Production and Measurement.

Invitrogen Human IL-6 Cytoset TM96 wells of microtiter plate were coated with 10ul of Anti-human IL-6 antibody in 10ml of coating buffer (PBS, pH 7. 4) and incubated overnight. Afterwards, it was blocked with 200 µl of 0. 5% BSA for 1hr at room temperature. Samples (50ul) and standards (recombinant human IL-6, 100µl) were loaded in duplicates followed by the addition of 50µl detection antibody (Anti-Human IL-6 Biotin 0. 025mg/0. 125ml) and incubated for 2hrs at room temperature. Streptavidin –HRP (0. 25ml) was added with continual shaking (30mins room temperature) and the reaction was visualized by the addition of 50 µl Tetramethlybenzene (TMB) substrate for 30mins. The reaction was stopped with 100 µl Sulphuric Acid (H2SO4) and absorbance at 450nm was measured using ELISA plate reader. Plates were washed five times with washing buffer (PBS, pH 7. 4 containing 0. 5ml tween 20) after each step. Invitrogen Human TNF-αCytoset TM96 wells of microtiter plate were coated with 10ul of Anti-human TNF-α antibody in 10 ml of coating buffer (PBS, pH 7. 4) and incubated overnight. Afterwards, it was blocked with 200 µl of 0. 5% BSA for 1hr at room temperature. Samples (50ul) and standards (recombinant human TNF-α, 100 µl) were loaded in duplicates followed by the addition of 50 µl detection antibody (Anti-Human TNF-α Biotin 0. 025mg/0. 125ml) and incubated for 2hrs at room

temperature. Streptavidin –HRP (0. 25ml) was added with continual shaking (30 mins room temperature) and the reaction was visualized by the addition of 50 µl Tetramethlybenzene (TMB) substrate for 30 mins. The reaction was stopped with 100 µl Sulphuric Acid (H2SO4) and absorbance at 450nm was measured using ELISA plate reader. Plates were washed five times with washing buffer (PBS, pH 7. 4 containing 0. 5ml tween 20) after each step.

Western blotting

Two separate proteins from the cell lysates to test for PAR-2 and AHR were extracted with 0. 4% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis on separate cartridges. The proteins were transferred onto separate nitrocellulose membrane (X-cell 11tm Blot) for 2hrs at 25 volts. Both membranes were placed in a blocking solution and incubated at 4oc overnight to block non-specific sites. The membranes were washed 6 times at 5 mins interval with Tris buffered saline (TBS). One of the membranes was incubated with a PAR2 monoclonal primary antibody (SAM II, UK) at a 1: 1000 dilution for 2hrs. This antibody is directed to amino acid SLIGKV whereas the other membrane was incubated with AHR antibody (RPT1). Another washing period was carried out 6 times at 5 mins interval with TBS for both membranes. Both membranes were incubated with the secondary antibody anti-mouse IgG conjugated to Horse Radish Peroxide at a 1: 2000 dilution factor for 1hr at room temperature. The membrane was developed using ECL western blotting antibody detection reagent and developed with Chemiluminescence (ECL plus).

Statistics

Data illustrated represent mean +/- SEM. Statistical comparison was analysed using two-tailed test and One-way ANOVA analysis. Results were considered significant when the P value is less than 0. 05.

RESULTS

Human Monocytes exhibit PAR-2 expression

Expression of PAR2 was analysed in freshly prepared non-differentiated cell lines U937 and THP1 which was derived from a patient with acute myeloid leukaemia, which were bound to a SAM II PAR-2 monoclonal antibody with the use of a flow cytometry. PAR-2 expression was detected and the percentage expression of PAR-2 in U937 Non- Stimulating Cell was 7. 49% where as in THP1, it was 11. 13%. PAR-2 expression of the THP1 cells was shown to be higher than the U937 cells figure 1a-1b. This is to confirm that human monocytes express PAR2. Both monocytes were incubated with SLIGKV, Reverse peptide, LPS (100ng/ml), SLIGKV+LPS and PMA/ionomycin (50ng/500ng) to examine the effect each stimuli has on PAR2 expression. According to the data presented in Figure 1. There was no increase in both the surface and intracellular expression of PAR2 of these cells. The experiment was performed only on undifferentiated monocytic cells. PMA/ionomycin appears to reduce PAR2 surface expression in both THP1 and U937 cells. The same observation was noticed in the cell stimulated with LPS. Due to time constraint, further experiment couldn’t be carried out on these differentiated cells in PMA/ionomycin for three days or more to observe any PAR-2 expression level changes. FACS analysis of AHR expression was

also examined on these cells using the anti mouse AHR PE antibody which was commercially made available. But unfortunately, it does not recognise human AHR Figure 1. The next step was to carry out a western Blot test to observe both PAR2 protein expression and AHR expression in both U937 and THP1 (only U937 data shown. THP1 data not shown). No bands were detected for U937, THP1 and BMM except for the positive control HEPG2 Figure 2. This could be due to inadequate number of monocytes or experimental problem with the procedure to detect the protein levels in each cell lines. In conclusion, procedure needs to be repeated with the use of a higher volume of monocytic cell.(B)(A)Fig. 1. FLOW CYTOMETRIC ANALYSIS OF PAR-2 AND AHR EXPRESSION IN BOTH U937 and THP1 HUMAN MONOCYTIC CELLS. Freshly cultured undifferentiated monocytes (1x106ml) were analysed using specific monoclonal antibody for PAR-2 (SAM II), IgG isotype matched control antibodies and Anti mouse AHR PE (FACS). These cells were stimulated with LPS (100ng/ml), SLIGKV, PMA/ionomycin (50ng/500ng) and reverse peptide to detect their influence on PAR-2 expression. (a) Human monocyte U937 cells FACS data (b) Human monocyte ThP1 cells FACS data(B)66. 4 KDA60 KDAFig 2. Monocyte lysates (protein 10ul) were isolated from THp1 & U937 cell which was used for a western blot analysis as described in " Materials and Methods" to detect the presence of PAR-2 proteins and AHR expression with the appropriate antibodies.

Surface and Intracellular expression of cells stimulated by TCDD

Each monocytic cell lines were stimulated with a dose response of TCDD (1, 10, 100 nm), LPS (100ng/ml) and PMA/ionomycin (150nm) to observe both

the surface and intracellular expression of PAR-2 which is presented in Figure 3. According to the data, the dose response of the TCDD appears not to alter the surface and intracellular expression levels of PAR2 in U937 and THP1 cells. The LPS activation of both cells was also shown to be poor. This indicates that the toll like receptors expression response was decreased in this non-differentiated cells and this might actually have an effect on the cells in expressing PAR-2. According to the data, its shows that PMA/ionomycin up-regulates PAR-2 expression intracellularly of both the U937 and THP1 cells whereas on the surface, it down-regulates PAR2 expression. In comparing the cell surface expression of PAR2 in U937 and THP1, U937 tends to express more PAR2 compared to THP1. However, THP1 showed more PAR2 expression than U937 cells intracellularly. These results indicate that these two lines might have originated from one source but might still exhibit different immune response. The statistics presented was only for one experiment. More experiments need to be performed on both non-differentiated and differentiated monocytic cells to observe the par-2 expression on both cell surface and intracellularly to conclude on the PAR2 expression of these cells.(B)(A)Fig. 3. Intracellular and surface expression of PAR-2 on undifferentiated monocytes stimulated with TCDD and LPS. (A) Undifferentiated U937 and THP1 cells were analysed for par-2 surface expression (B) Undifferentiated U937 cells and THP1 were analysed for intracellular expression of PAR-2. The data shown is only for one experiment therefore no statistics available.

Effect of TCDD on CD14+ cells, GMCSF and MCSF cytokine production

CD14+ cells was isolated and cultured at 2. 5x105 ml in 96 well plates in the presence of human GM-CSF (M1) and M-CSF (M2). After 6 days, the cells were viewed under the microscope and the MCSF (M2) cells consist of green intracellular structure with circular shape whereas in the GMCSF (M1) wells, it was observed that cells seem to be numerous and circular in shape. Cells were then stimulated with TCDD (100 nM) and LPS (100 ng/ml) after 24 hours. Cytokine secretion of cells were investigated following exposure to TCDD and displayed in Figure 4a. The inflammatory response of CD14+, GMCSF and MCSF when given a dose response of LPS were assessed by measuring the levels of TNF-α. According to results, it was shown that GM-CSF (M1) produces more TNF-α than MCSF & CD14+ in response to LPS compared with the control ($p < 0.05$). Dose response of TCDD (1, 10, 100 nm) was added to these cells to observe the effect on cytokine secretion. According to the result, TCDD appears to significantly reduce the TNF-α secretion in M1 and M2 cells. Another experiment was carried out to see if these cells produce IL-6. According to data, M1 cells seem to produce more IL-6 than M2 and CD14+ following activation by LPS. However, a dose response of TCDD appears not to significantly alter IL-6 production in either M1 or M2. In contrast, TNF-alpha and IL-6 were produced rapidly by the non-stimulating cells of both M1 and M2 when activated by LPS but was reduced significantly by TCDD. This result shows that the cytokine levels induced by TCDD response remained unchanged during the TCDD concentration levels (1nm, 10nm and 100nm). Fig. 4. Cytokine Analysis and differentiation of

macrophages. Human cells (2 x 106 ml) was cultivated with TCDD (1, 10, 100 µl/ml) and LPS (100ng/ml) for 24 hours and production of TNF-alpha & IL-6 was determined by cytokine ELISA as described in " materials and Methods" in supernatants. (A) M1 and M2 monocytes were stimulated with GMCSF (20ug/ml) and MCSF (10ug/ml) and cytokine secretion was examined for both IL-6 and TNF-a with the CD14+ subset acting as a control. (** p <0. 05) versus control.

TCDD inhibits cytokine secretion in non-differentiated monocytic cells

Both U937 and THP1 cell lines which are derived from a patient with acute leukaemia were centrifuged and cultured at 1 x 106ml. The inflammatory response of both monocytes was assessed by measuring the levels of TNF-α & IL-6 cytokine production with the use of ELISA after 24 and 48 incubation. Before this procedure, cells were stimulated with TCDD (1, 10, 100nm) & LPS (100ng/ml) to observe the effects both factors have on cytokine emission in these cells. As shown in Figure 5, Non-differentiated U937 cells produced low levels of TNF-α and IL-6 cytokines and also displayed poor LPS activation. When given a dose response of TCDD, it appeared not to significantly alter the TNF-α secretion both in 24 hrs and 48 hrs. However, TCDD was shown to increase the levels of IL-6 production during the 24 hr analysis and the results when calculated with a T-test appeared to be significant. This might indicate that TCDD plays a role in the alteration of IL-6 production in non-differentiated U937 cell lines but cannot regulate TNF-α production. In conclusion, procedure needs to be repeated to confirm observed findings in the IL-6 production of U937. Non-differentiated THP1 monocytes were

examined for both TNF-alpha and IL-6. They cells were able to produce cytokine when activate by LPS. However, the LPS activation was poor in the IL-6 data but was effective in the TNF-alpha. THP1 cells were not able to produce substantial amount of IL-6 and TNF-alpha. The TCDD dose response did not alter the cytokine secretion of both TNF-alpha and IL-6 secretion. In summary, TCDD was shown to reduce cytokine production and remained unchanged throughout both in the 24 and 48 hrs. duration for TNF-alpha and IL-6 production for THP1 cellsBoth cell lines need to be differentiated further into macrophages and stimulated with a dose response of TCDD to examine the effect it has on the cytokine production levels in both TNF-alpha and IL-6 and also compared with the non-differentiated cells to observe any changes. Fig. 5. Cytokine analysis of non-differentiated monocytes. Cytokine secretion were measured by ELISA as described in " Materials and Methods" for both 24 hrs& 48 hrs after stimulation with TCDD (1, 10, 100ul) and LPS (100ng/ml). (A) non-differentiated U937 cell data for TNF-alpha cytokine secretion. (B) Non-differentiated U937 cell data for IL-6 cytokine secretion. (C) Non-differentiated ThP1 cell data for TNF-alpha cytokine secretion. (D) Non-differentiated THP1 cell data for IL-6 cytokine secretion. The graph show mean for four independent experiments. Experiments were carried out in triplicates. The error bars show the standard error of the mean. ** P value < 0. 05 versus control

Cytokine secretion in differentiated cells and response to TCDD stimulation

Fig. 6. Cytokine analysis of differentiated monocytes. Cytokine secretion were measured by ELISA for both 24 hrs& 48 hrs after stimulation with TCDD

(1, 10, 100ul) and LPS (100ng/ml) after being differentiated by pma/ionomycin (150nm) into macrophages. (A) Differentiated U937 cell data for TNF-alpha cytokine secretion. (B) Differentiated U937 cell data for IL-6 cytokine secretion. (C) Differentiated ThP1 cell data for TNF-alpha cytokine secretion. (D) Differentiated THP1 cell data for IL-6 cytokine secretion. The graph show mean for four independent experiments. The error bars show the standard error of the mean. ** P value < 0. 05 versus controlThe effect of TCDD on TNF-alpha and IL-6 expression was investigated in differentiated human U937 and THP1 macrophages. They are both grown in a growth cultured suspension and do not adhere to the plastic surface of the culture plates. Both cells were treated with PMA (150nm) for 6 days to differentiate into macrophages. The level of secretion of both TNF-alpha and IL-6 in the culture supernatant was measured after being stimulated in the presence of 1, 10 and 100nm of TCDD for both 24 hrs and 48 hrs. The data is presented in Figure 6 for both U937 and THP1 cells. According to the data, activation of the differentiated macrophages by the LPS resulted to an increase of TNF-alpha and IL-6 production in both cells. In the U937cells, LPS activation was effective. However, over a dose response of TCDD caused a down regulation of TNF-alpha production. During the IL-6 cytokine production, TCDD tend to significantly upregulate cytokine production when compared to the non-stimulating cells but does not change over the dose range (1nm-100nm) in both 24 and 48 hrs time periods. This observation was also noticed in the non-differentiated U937 cells. These results indicate that TCDD induces a cytokine response but it does not change over a dose response range. In THP1 cells, the cells were shown to secrete high levels of TNF-alpha when

stimulated with LPS. Over a dose response of TCDD, there was not an up regulation of cytokine production between the 24 to 48 hrs. These results indicate that although TCDD induces cytokine response in these cells however, it does not change its cytokine production level depending on its concentration levels. When compared to the IL-6 data, THP1 cells produced less amount of IL-6 cytokine secretion both when stimulated with LPS and in non-stimulated cells. The TCDD dose showed to not have an effect on THP1 cells both in regulation and cytokine quantity production. In contrast between both U937 and THP1 cells, THP1 produces more TNF-α than U937 and the cytokine production are not affected by TCDD dose response whereas during the IL-6 cytokine production, U937 produces more IL-6 than THP1. Additionally, TCDD dose response tends to significantly upregulate IL-6 cytokine production in U937 cells. In comparison with the non-differentiated cell lines, both the U937 and THP1 cells produce the double amount of cytokines when differentiated than non-differentiated cells for both IL-6 and TNF-α production. DISCUSSIONStudies involving the link between the regulation of PAR2 and innate immune effector function by an AHR agonist have not been established yet. This study was demonstrated to prove the hypothesis which is to show the AHR role in PAR2 regulation or vice versa with the use of a human innate effector cell such as monocytes and macrophages. Involvement of PAR2 in pro-inflammatory immune response has been shown in previous studies (Johansson et al, 2005: Colognato et al, 2003). Most of these studies have been performed on monocytic cell lines. So far, Human monocytes have been shown to express both PAR2 and AHR by different pathways according to previous studies (Johansson et al, 2005:

Hayashi et al, 1995). Studies have shown roles of both PAR2 and AHR in innate macrophage effector function. The innate immune system is the first line of defence against foreign antigens and monocytes have been described as the main producers of both pro and anti-inflammatory mediators. The first experiment carried out was to look at PAR2 and AHR expression through FACS analysis. According to the experiment, monocytic cell lines U937 and THP1 were able to express PAR2 which support a previous study of the ability of the cell lines to express a discrete cell surface expression of express PAR2 (Johansson et al, 2005). However, this PAR2 expression on these cells was significantly reduced by PMA/ionomycin and LPS. This might suggest that these cells were unable to express high PAR2 expression due to the PMA/ionomycin causing a prominent alteration of cell morphology and membrane expression of the cells or factors released from cell in response to external stimuli can down-regulate PAR2 expression. These non-differentiated cell lines were further examined for both surface and intracellular PAR2 expression stimulated with a dose response of TCDD through FACS. This was performed to investigate whether PAR2 expression is influenced by AHR. Unfortunately, the PAR2 expression remained unchanged. The results provided were for only one independent experiment. The procedure needs to be repeated in both non-differentiated cells and differentiated monocytic cells to conclude whether AHR regulate PAR2 expression on these cells both in non-differentiated and differentiated. AHR analysis was performed on this cell with the use of FACS after being cultured with the anti-mouse antibody. Apparently, the antibody used does not recognize human AHR. Therefore, the next step was to perform a western

blot test with the use of anti-mouse antibody for AHR and SAM II for PAR2 expression. According to the result, there was no band detected for U937 and THP1 cells in for both AHR and PAR-2 but there was a band detected for the control HepG2 (U937 data shown only). Previous studies have shown AHR to be expressed by monocytes with the use of polyclonal anti-AHR antibody PA3-513. Although the AHR proteins levels of the cell lines were lower than the control subset HepG2 cells (Komura et al, 2001). This is to conclude that monocytes display PAR2 expression and AHR but there is no evidence from other studies showing that they are both linked together in a signalling pathway. Another approach was taken by looking at the cytokine production of these monocytes both non-differentiated and differentiated. U937 and THP1 cells are the ideal model for study in connection to monocytes function and cytokine production. Both U937 and THP1 are the cells used for the initial starting point of experiment in which they were cultured in growth medium and shown to divide indefinitely in vitro. U937 cells are of tissue origin and THP1 cells are of blood leukemic origin at less mature stage (Baek et al, 2009). Both undifferentiated and differentiated U937 cells and THP1 cells were stimulated with a dose response of TCDD and LPS. LPS was used as a control to check that they cells are viable and also are producing cytokines whereas TCDD which is an AHR agonist was used to check for the effect on cytokine production in these cells. Firstly, the non-stimulating cells of the U937 cells was shown to produce a substantial amount of IL-6 and TNF-alpha cytokine production but the TCDD dose response significantly regulated cytokine production in Il-6 but not in TNF-α. The non-differentiated THP1 cell was shown to have low amount of cytokine

production in both TNF-alpha and IL-6. The LPS activation of both undifferentiated cells was also reduced due to the maturation stage of these cells These results indicate that there are several factors which might inhibit the cell activation at this stage such as the toll like receptors on the cell surface was not able to respond to external stimuli due to decrease in expression. Another factor can be the Nf-Kb transcription factor accumulation in the cytoplasm of the cells in which they are unable to induce cellular events. The NF-kb initiates the cells to respond to LPS. Once activated, the Nf-Kb moves from the cytoplasm to the nucleus of the cell (Takashiba et al, 1999). The TCDD dose response was shown to not have an effect on the cytokine change in both TNF-α and IL-6 production in the non-differentiated cell lines. Therefore, the cells were further differentiated for 6 days in PMA/ionomycin to observe for the AHR response by the AHR agonist TCDD and LPS stimulation. The LPS activation in these cells was effective which lead to cytokine production. This might be due to the effect of the LPS on the TLR-4 receptors on this cell at their differentiation stage and cell composition which supports a previous study about the ability of macrophages to acquire specialized receptor in recognizing foreign bodies once differentiated (Zhenyu, Q. 2012: Schull et al, 2010). The THP1 cells was shown to produce more TNF-α than U937 in response to TCDD which supports a previous study which showed that TCDD was able to increase TNF–alpha production in these cells (Cheon et al, 2007). However, TCDD had no effect on the regulation of cytokine production in the THP1 cells both in 24 and 48 hrs duration. In U937 cells, another observation was seen on the experimental data which was also observed in the non-differentiated U937

cells. TCDD was shown to up regulate IL-6 production for the 1nm and 10nm dose response but it remained unchanged in 100nm. TCDD dose response might be involved in IL-6 cytokine production of U937 cells. In summary TCDD was found to enhance TNF-alpha production in differentiated human THP1 but had no effect on the IL-6 production of these cells whereas TCDD was shown to significantly influence the TCDD response in IL-6 for U937 but not in TNF-α cytokine production. In conclusion, It has been shown that AHR is expressed based on the effects of TCDD on cell numbers of monocytes and AHR levels have been shown to increase depending on the response of the TLR ligands expressed on the cell surface of the cells. According to this study, AHR expression was induced better in the differentiated cell lines than the non-differentiated cell lines. Factors which might have contributed to this are toll like receptors and NF-KB transcription factor (Stockinger et al, 2011: Hayashi et al, 1995)Findings observed with the use of human monocytic cell lines to detect PAR2 expression and AHR were repeated this time with the use of M1 and M2 cells. FACS and western blot analysis was not performed on M1 and M2 to look at PAR2 and AHR expression. Cytokine secretion of these cells was looked at to examine its physiological response. Both Il-6 and TNF-alpha secretion was looked at for CD14+ cells which was stimulated and differentiated into M1 and M2 by growth stimulating factors such as GMCSF and MCSF. GM-CSF and MCSF have been shown to increase cell survival, cell proliferation and cell activation in cultured cells of monocytes and macrophages. They also enable the monocytes for an increase response to external stimuli such as LPS (Hamilton, J. 2008). From the results, we can conclude that GMCSF produces more TNF-α and IL-6 than MCSF and CD14+

when stimulated with LPS. LPS is required for GMCSF and MCSF to be activated in these cells to secrete significant level of cytokines as a form of control. Culturing of LPS with GMCSF enabled the cells to produce pro-inflammatory cytokines such as TNF-α and IL-6 whereas MCSF cultured cells produces less TNF-alpha and IL-6 (Hamilton, J . 2008). And this is crucial because GMCSF is involved in inflammatory response in cells. The cells were stimulated with a dose response of TCDD to examine the effect on the cytokine production. TNF-α cytokine secretion for GMCSF was reduced significantly by TCDD and the same result was seen for both MCSF and CD14+ compared to the LPS control. These results suggest that TCDD inhibits the cytokine secretion of these cells and also does not alter cytokine production levels based on dose response in variable concentration (1nm-100nm). In conclusion, Both GM-CSF and MCSF are growth factor for macrophages and also an activator of monocyte/macrophage cell lineage. They are also able to induce a HLA class II expression on the cell surface of these cells and cytokine synthesis (Haworth et al, 1991: Chantry D, 1990). There might be a possibility in which the class II expression of these cells might be a factor in which the TCDD dose response variable concentration can alter the cytokine production levels. With all this evidence gathered, it has been shown that PAR2 expression is not regulated by AHR. Although in some cases, AHR was shown to inhibit cytokine production in these cells compared to the control. This might indicate that TCDD has a strong down regulatory effect in these cells for the production of cytokines. We cannot say at this point that PAR2 expression cannot be controlled by AHR or that a link between the two is non-existent but with the right experimental

approach or research, a new finding can be discovered which could establish a link between the two factorsFuture studies might be advantageous in this field if other monocytic cell lines are examined for both PAR2 expression and AHR in both human and murine models. TCDD has been shown to be the most potent ligand for AHR but looking at the response of AHR by other AHR agonist can also give us an insight on the cytokine production or PAR2 expression of these cells (kobayashi et al, 2008) In conclusion, PAR2 and AHR have been shown to play a major role in the human innate effector cells but a link showing that the two are involved in the same pathway was not established in this study.

Acknowledgement

I would like to thank Prof. John Lockhart and Anne Crilly for their support and assistance they gave to me during the duration of my honours project and literature review. I would like to thank my Family especially my mother for her continuous words of encouragement, support and advice in both my academics and life.